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BAKER BOTTS LLP		EXPRESS MAIL LABEL No	DATE Sept 21, 2001
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35.U.S.C. 371		ATTORNEY'S DOCKET NO 34585-A-PCT/USA 070050.1667	
		U.S. APPLICATION NO To be assigned 09/937165	
INTERNATIONAL APPLICATION NO PCT/US00/06862	INTERNATIONAL FILING DATE 15 March 2000	PRIORITY DATE CLAIMED 19 March 1999	
TITLE OF INVENTION IMPROVED EXPRESSION VECTOR FOR CONSISTENT CELLULAR EXPRESSION OF THE TET-ON REPRESSOR IN MULTIPLE CELL TYPES			
APPLICANT(S) FOR DO/EO/US The Trustees of Columbia University in the City of New York			
<p>Applicant herewith submits to the United States Designated /Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I). <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> has been transmitted by the International Bureau. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210) <ol style="list-style-type: none"> <input checked="" type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> have been transmitted by the International Bureau <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. <input type="checkbox"/> have not been made and will not be made. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). <p>Items 11. to 16. below concern other document(s) or information included:</p> <ol style="list-style-type: none"> <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409) <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. <input type="checkbox"/> A substitute specification. <input type="checkbox"/> A change of power of attorney and/or address letter. <input type="checkbox"/> Other items or information: <ol style="list-style-type: none"> <input checked="" type="checkbox"/> a copy of the International Search Report (PCT/ISA/210) <input type="checkbox"/> a copy of the International Preliminary Examination Report (PCT/IPEA/409) Petition to Revive + check for \$620.00 PCT application No. PCT/US00/06862 was published in English under publication number WO 00/55310 on August 24, 2000. Return receipt postcard 			

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Fisher et al.
Serial No. : Not yet assigned Examiner : Not yet assigned
Filed : Not yet assigned Group Art Unit: Not yet assigned
For IMPROVED EXPRESSION VECTOR FOR CONSISTENT CELLULAR
EXPRESSION OF THE TET-ON REPRESSOR IN MULTIPLE CELL
TYPES

PRELIMINARY AMENDMENT AND PRIORITY CLAIM

Express Mail Label No. EF321683201US

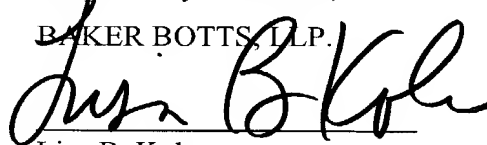
Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination please amend the specification of the above-identified patent application by replacing the first paragraph with the paragraph: "This is a national phase application based on International Patent Application No. PCT/US00/06862, filed March 15, 2000 and published in English on September 21, 2000, which claims priority as a continuation in part to United States Patent Application No. 09/268,303, filed March 15, 1999."

Respectfully submitted,

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**IMPROVED EXPRESSION VECTOR
FOR CONSISTENT CELLULAR EXPRESSION
OF THE TET ON REPRESSOR IN MULTIPLE CELL TYPES**

This application claims priority and is a continuation-in-part application of U.S. Serial No. 09/268,303, filed March 15, 1999, the contents of which is hereby incorporated by reference.

The invention disclosed herein was made with Government support under Grant No. CA 35675 from the National Institutes of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Background of the Invention

Since the first report by Gossen and Bujard (Gossen and Bujard, 1992) and subsequent documentation of a variant form (Gossen et al., 1995), the Tetracycline (Tc)-regulated system, has been broadly adopted and is widely acknowledged as the method of choice, in experiments requiring inducible expression of genes of interest. In its originally reported form, the system employs two plasmids. One expressing the tTA or rtTA cDNA (henceforth jointly referred to as TA), a fusion protein of the bacterial Tc-repressor, fused to the C-terminal acidic activation domain of the Herpes Simplex virus (HSV), VP16 transcriptional transactivator. The second plasmid enables cloning of a cDNA of interest downstream of a heptamerized Tc-operator transcription regulatory DNA sequence, fused to a DNA element providing basal promoter

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activity, derived either from the CMV IE or HSV thymidine kinase promoters. Establishing a cell line having Tc-regulatable expression of the gene of interest involves a two step process. In the first, a cell line stably
 5 expressing the TA cDNA is established and identified by clonal selection and expression analysis through transient transfection with a Tc-responsive reporter. In the second step, the gene of interest cloned under control of the Tc-responsive element is introduced into the cell line made
 10 in the previous step and a second round of selection is performed to identify clones displaying Tc-responsive inducibility of the cDNA (Gossen and Bujard, 1992; Gossen et al., 1995). The Tc-regulated system has effectively overcome several drawbacks seen in earlier systems which showed high
 15 basal levels of expression, poor responsiveness and toxicity of the inducing agent. The Tc-inducible system is in addition, able to achieve induction over ranges of several orders of magnitude in a graded manner, responsive to varying levels of inducer. Furthermore, the system is extremely
 20 versatile and amenable to several types of modifications, permitting the study of the role of a particular gene, or combinations thereof, in a wide variety of cell types of interest. The potential to use this system in medical applications including gene therapy protocols and
 25 pharmacological small molecule screening are areas of active investigation. Its versatility has enabled adaptation to situations requiring inducible gene expression in a tissue specific or generalized manner in animal or plant models, opening new avenues to study gene function *in vivo*.

30 The Tc-inducible expression system has been modified in several ways, in attempts to improve performance or tailor it to specific needs. Autoregulatory control was achieved by placing both the tTA as well as exogenous cDNA under control
 35 of Tc-operator sequences (Shocket et al., 1995), which reportedly permitted regulation of available tTA levels only

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on induction and thereby increased overall performance in terms of inducibility and frequency of positive clones obtained. Single plasmid vectors containing the tTA sequence and gene of interest in opposite orientations have been developed to obviate the need for multiple rounds of clonal selection (Baron et al., 1995; Schultze et al., 1996; Weinmann et al., 1994). Overcoming a sometimes considerable barrier of introduction of DNA into transfection recalcitrant cells has been made possible through the development of retroviral vectors for delivery of both components of the system in either a single or combination of two separate viruses (Bohl et al., 1997; Hofmann et al., 1996; Kringstein et al., 1998; Paulus et al., 1996; Rossi et al., 1998). Several promoters have been used to enable generalized or tissue specific expression of tTA in plants (Weinmann et al., 1994) or animals (Efrat et al., 1995; Fishman et al., 1994; Furth et al., 1994; Hennighausen et al., 1995). Modification of the Tc-operator containing plasmid to reduce leaky expression or reduce the effects of integration site has been attempted. Strategies toward this end include Epstein Barr virus (EBV) replication origin based vectors that are maintained episomally (Jost et al., 1997), modified basal promoters to reduce uninduced expression (Hoffmann et al., 1997) and incorporation of sequences that prevent interference from adjoining elements at the site of integration (Hennighausen et al., 1995; McKnight et al., 1992; Stief et al., 1989).

The original report and several other studies have documented potential pitfalls and have provided troubleshooting strategies using the Tc regulated system (reviewed in Blau and Rossi, 1999; Gossen et al., 1994; Shockett and Schatz, 1996)). However, anecdotal evidence non-rigorously documenting failure to establish cell lines that show any significant levels of expression or inducibility of the exogenously introduced gene (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995) exists. Drawing upon previous

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experiences using expression constructs with strong viral promoters based on CMV or SV-40 derived sequences, extinction of expression of transactivator function could be a potentially significant factor encountered in the inability to establish Tc-responsive cell lines. This might be of special relevance in cells having a relatively slow growth rate and/or the potential to differentiate, making them particularly sensitive to this phenomenon, since changes in cell physiology could affect the activity of exogenously introduced viral promoter constructs. The time lapsed between establishing the initial TA expressing clone and identification of cell lines inducibly expressing the gene of interest, is of a sufficient duration, during which the host cell possibly stops supporting CMV promoter enhancer expression, resulting in the shutdown of TA expression. Despite the recent introduction of retroviral vectors that enable single step and therefore relatively quick selection of positive clones, several of these also depend on viral promoters for expression of one or more elements and are therefore also prone to similar problems. The construction of a specific retrovirus is in itself time consuming and a not as yet routine procedure in many laboratories, compared to transfection or electroporation of plasmid DNA into cells. Based on these factors modification of the existing construct for rtTA cDNA expression was done by placing it under the regulation of the human Protein Translation Peptide Elongation Factor-1 α promoter (EF-1 α). This gene has a housekeeping function in all cells and has been documented to be expressed to relatively high levels. More importantly, due to its indispensable housekeeping function in all cells, Protein Translation Peptide Elongation Factor-1 α promoter (EF-1 α) expression is consistent from a temporal viewpoint, relatively insulated from changes in cell physiology and is cell type independent (Goldman et al., 1996; Kim et al., 1990; Wakabayashi-Ito and Nagata, 1994). Utilization of this construct in cells lines derived from diverse human tissues

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enabled the successful construction of Tc-regulatable lines
in every case attempted so far. This modified vector will
not only be of general utility but will be especially useful
in cases where difficulties have been previously experienced
5 in successfully establishing Tc-responsive clones.

Summary of the Invention

The present invention provides a cell comprising the vector set forth above. The present invention further provides that the cell is from a cell line. The present invention further provides that the cell line is HeLa (human cervix), HO-1 (human melanoma), MCF-7 (human breast), PC3 (human prostate) or DU-145 (human prostate).

The invention also provides an animal comprising the vector set forth above. This invention also provides an animal which comprises a cell which comprises Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor-1 α promoter. This invention also provides the animal includes but is not limited to a mouse.

The present invention provides a method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising: (a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion (b) generation of Protein Translation Peptide Elongation Factor-1 α promoter vector, by restriction enzyme digestion (c) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs (d) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by Klenow fragment mediated blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' XbaI end of Protein Translation Peptide Elongation Factor -1 α promoter vector and (e) blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor -1 α promoter vector expressing reverse tetracycline

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controlled transactivator.

- This invention provides the fragment includes but is not limited to an *Eco* RI-*BAM* HI fragment, the mammalian expression vector includes but is not limited to pCDEF3, cloning is at the 5' *Eco* RI and 3' *BAM* HI of the insert and the ligation is at the 5' *Eco* RI site and the 3' *Xba*I site of pCDEF3.
- 10 This invention provides a method of screening pharmacological products using the vector. Finally, this invention provides a method for monitoring inducible gene expression in a tissue specific of generalized manner using the vector.

Brief Description of the Figures**Figure 1**

Plasmid map of Protein Translation Peptide Elongation Factor-1 α expression construct: The map shows individual component elements of the vector including the rtTA ORF, human EF-1 α promoter, Bovine growth hormone (BGH) polyadenylation (poly A) signal and partial multiple cloning site retained from the vector pCDEF3 (Goldman et al., 1996) after cloning. The Neomycin resistance marker (NeoR) flanked by the SV40 promoter and poly A signal, Ampicillin resistance marker (AmpR) for bacterial propagation and selection and some reference restriction site are also shown.

Figure 2

Luciferase assay to test activity of the Protein Translation Peptide Elongation Factor -1 α promoter vector: Extracts from human HO-1 melanoma cells transiently co-transfected with the original (bars marked pUHD 17-1neo) or modified (bars marked EF1p Tet on) rtTA expression vectors and the Tc luciferase reporter pUHC 13-3 were quantitated for luciferase activity. These extracts were prepared from cells treated without the inducer (-Dox) or with (+Dox). Treatment with inducer was for 48h as described in materials and methods.

Figure 3

Luciferase assay to select Tc-inducible clones: Panels show quantitation of luciferase assays from individual Neomycin resistant clonally isolated cell lines of human prostate (DU-145 and PC3), cervical (HeLa), breast (MCF-7) and melanoma (HO-1) tumor origin. Each stable clone was transiently transfected with the Tc luciferase reporter pUHC 13-3 in the absence (-Dox) or presence (+Dox) of inducer. Extracts prepared from these cells were assayed

for luciferase activity to identify clones showing adequate levels of inducibility for each cell type as described in material and methods.

5 **Figure 4**

Northern blot analysis of individual Tc responsive clones expressing regulatable Mda-7 or Jun B cDNAs: Autoradiographic detection of levels of induced RNA message levels expressed in clonally selected cells stably
10 transfected with the Mda-7 (A) or Jun B (B) cDNAs under regulation of Tc, probed with respective radiolabelled cDNA probes after transfer to nylon membranes. Each similarly numbered sample was derived from the same clone without induction [1-17(A) and 1-9 (B) or after addition
15 of inducer, 1'-17' (A) and 1'-9' (B)].

Figure 5

Plasmid name: pEF1ptTA. **Plasmid size:** 7.02kb.
Constructed by: Gopalkrishnan et al., Nucleic Acids
20 **Research 27:** 4775-4782 (1999) & refs. therein.
Construction date: January 2000. **Comments/References:** The ORF for the tetracycline repressor VP16AAD fusion driven by Human EF-4 alpha promoter. Source of tTA was pUHD15-1, isolated as an EcoRI/BamHI fragment and cloned into the 5'
25 EcoRI-BamHI (blunted) 3' XbaI (blunted) site of pCDEF3.

Detailed Description of the Invention

The present invention provides a vector comprising an Protein Translation Peptide Elongation Factor -1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor -1 α promoter. In an embodiment the vector is a plasmid. In another embodiment the vector is as set forth in figure 1.

The present invention further provides a cell comprising the vector set forth above. In an embodiment the cell is from a cell line. In a further embodiment the cell line is HeLa (human cervix), HO-1 (human melanoma), MCF-7 (human breast), PC3 (human prostate) or DU-145 (human prostate).

The invention also provides an animal comprising the vector set forth above. An embodiment of this invention the vector has been introduced into the animal or an ancestor of the animal at an embryonic stage. The animal includes but is not limited to a mouse.

This invention also provides an animal which comprises a cell which comprises Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor -1 α promoter.

The present invention provides a method of generating a A method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising: (a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion (b) generation of Protein Translation Peptide Elongation Factor -1 α promoter vector, by restriction enzyme digestion (c) directional cloning of reverse tetracycline controlled

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transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs (d)directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by Klenow fragment mediated blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' XbaI end of Protein Translation Peptide Elongation Factor -1 α promoter vector and (e)blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor -1 α promoter vector expressing reverse tetracycline controlled transactivator.

In accordance with the method of the invention, the fragment includes but is not limited to an Eco RI-BAM HI fragment, the mammalian expression vector includes but is not limited to pCDEF3, cloning is at the 5' Eco RI and 3' BAM HI of the inserts and the ligation is at the 5' Eco RI site and the 3'XbaI site of pCDEF3.

The present invention provides a vector which is directed to providing a consistent cellular expression of the tetracycline repressor in cells. Such a vector may be useful in situations requiring inducible gene expression in a tissue specific or generalized manner in animal or plant models. In one embodiment of the invention, pharmacological products are monitored to determine use in medical applications. In the preferred embodiment monitoring is of the gene changes associated with cellular process such as aging, cancer, development, differentiation and growth.

More specifically, methods which are well known to those skilled in the art can be used to construct a vector directed to providing a cellular expression of the tetracycline repressor in cells. These methods include in cell culture

Construction of Plasmids: An *Eco* RI-*Bam* HI fragment containing the rtTA open reading frame was isolated from pHUD17-1neo (Gossen et al., 1995). This fragment was cloned directionally into the mammalian expression vector pCDEF3 (Goldman et al., 1996) at the 5' *Eco* RI and 3' *Xba* I sites of the vector multiple cloning site to generate the final construct termed, EF1prtTA. Ligation of the 3' *Xba* I site of pCDEF3 and the *Bam*HI site of the fragment was possible after Klenow filling the overhangs to make them blunt-ended. This

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modified vector places the rtTA gene under direct transcriptional control of the human polypeptide chain elongation factor-1alpha promoter (EF-1a). Plasmids expressing the Mda-7 and Jun B cDNAs were constructed in pUHD 10-3 (Gossen and Bujard, 1992) by blunt cloning of isolated cDNA fragments into Klenow filled blunt vector followed by sequence analysis for confirmation.

Cell culture and derivation of stable cell lines: All cell lines used in this study were grown and maintained under standard conditions as previously described (Giang et al., 1996). Selection of stable clones expressing the rtTA cDNA using EF1prtTA was carried out in the presence of 500 to 1000 µg/ml G418 (Life Technologies Inc.) depending on the individual cell line. After the selection period, macroscopic visible colonies were picked, expanded and analyzed for activity by assaying for luciferase activity for rtTA expression or by Northern blot analysis of inducible cDNA such as Mda-7 or Jun B respectively.

Northern blotting: Total cellular RNA was resolved by denaturing formaldehyde agarose gel electrophoresis after isolation of RNA using an RNAeasy Kit (Qiagen). Transfer was done onto Hybond nylon membranes (Amersham) and probed with appropriately labeled cDNA probes for Mda-7 and Jun B.

Luciferase activity analysis: Luciferase assays were performed using a Luciferase Assay Kit (Promega) and quantitation was performed on a Turner Design TD 20/20 luminometer. Equal quantities of RNA were loaded on each gel following spectrophotometric estimation at 260 nm. Normalization of RNA levels between samples was confirmed by visualizing RNA on ethidium bromide stained gels. Normalization of luciferase activity was achieved by quantitating protein and adjusting the amount of extract to a fixed amount of protein.

RESULTS

Construction and initial testing of the EF-1a promoter based rtTA expression vector

5 Details of the cloning steps performed in construction of the EF-1 a promoter rtTA (EFlprtTA) expression vector is described in materials and methods and Fig 1. The protein expressed by this cDNA, a mutant form of the original bacterial Tc-repressor (Gossen et al., 1995), binds to and
10 activates transcription of genes downstream of Tc-operator binding elements, only when Tc is present. EFlprtTA was transiently co-transfected with the Tc-responsive luciferase reporter plasmid, pUHC 13-3 (Gossen et al., 1995), into HO-1 human melanoma cells to determine if the construct was
15 active. A parallel set of transfections was performed with the original CMV IE based construct, pUHD 17-1neo (Gossen et al., 1995) in the absence or presence of 1 µg/ml doxycycline(Dox). Cells were harvested 48 h after transfection and luciferase activity (Fig. 2) was determined
20 using a luminometric Luciferase assay system (Promega). As previously documented (Gossen et al., 1994; Gossen and Bujard, 1992; Gossen et al., 1995) transient assays poorly reflect the level of inducibility actually obtainable after final selection of stable clones, since basal levels of
25 expression change dramatically once plasmid DNA is integrated into chromatin. The initial experiments clearly demonstrated that the EFlprtTA expression vector was functional at comparable levels to the original pUHD 17-1neo construct in transient assays. Based on the positive activity obtained,
30 the EFlprtTA construct was utilized to establish stable lines expressing rtTA in HeLa (human cervical carcinoma), HO-1, (human melanoma) MCF-7 (human breast carcinoma) and PC3 and DU-145 (human prostate carcinoma) cancer cell lines.

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***Analysis of stable cell lines expressing the rtTA cDNA
under regulation of the EF-1 α promoter***

Cells were transfected with the EF1prtTA construct using Superfect transfection reagent (Qiagen) based on standard conditions recommended in the usage protocol. The efficiency of transfection, reflected by the number of clones obtained at the end of the selection period, varied with each cell line. Colonies were selected using Neomycin resistance conferred by the marker present within the construct. For every cell line, twenty-four Neomycin resistant colonies were isolated for further analysis. These individually selected clones were transiently transfected with the Tc-responsive luciferase reporter pUHC 13-3 (Gossen et al., 1995) to determine the presence and level of rtTA activity. Some cell lines used in this series of experiments had failed to generate Tc-responsive clones in previous attempts utilizing the CMV IE based construct pUHD 17-lneo (Gossen et al., 1995).

Results obtained in a screen to identify Tc-responsive clonal cell lines in HO-1, MCF-7, PC3 and DU-145 cells (Fig. 3) indicated that an average of at least two clones of the twenty-four or less clones finally analyzed per cell line, showed some levels of Tc-responsiveness. This frequency of positive clones is comparable, if not higher than that reported previously (Gossen and Bujard, 1992; Gossen et al., 1995). As mentioned above, the fold induction observed in the presence of Tc, though relatively low, is likely to be a reflection of leaky expression in uninduced conditions due to the transient transfection conditions used in this initial screen. Despite this leakyness, clones with high or low relative levels of inducibility were identifiable in every case and potentially usable cell lines were identified with relative ease.

**Functional analysis of stable clones expressing cDNAs
under inducible regulation of EF1prtTA**

In continuance of a major focus of our research involving analysis of the role of specific genes in melanoma differentiation, stable cells expressing differentiation associated genes including the transcription factor Jun B and the tumor suppressor Mda-7 (Jiang et al., 1996) under Tc-regulation were established in HO-1 melanoma cells. This human melanoma cell line has the ability to terminally differentiate in the combined presence of β -interferon and the Protein Kinase C (PKC) activator, mezerein. It is sensitive to culture conditions due to its capacity to differentiate, difficult to transfect and takes a relatively long time during selection to form visible colonies suitable for re-isolation as a clonal population of cells. HO-1 therefore presents an ideal proving ground for the efficacy of the EF-1 α promoter based vector. Using a suitable rtTA expressing cell line identified in the previous screen described above (Fig. 3), transfections were performed with the potentially Tc-regulatable Jun B and Mda-7 cDNAs cloned into the vector pUHD 10-3 (Gossen and Bujard, 1992). Colonies were isolated and individual clones were analyzed for expression and induction of Jun B and Mda-7 by Northern blotting. To determine the level of inducibility of individual clones, RNAs were isolated from each clone grown in the absence or presence of Dox. Northern blots, probed with Jun B and Mda-7 cDNA probes (Fig. 4) indicated that several positive clones had been obtained for each cDNA. As anticipated, varying degrees of clone dependent basal and inducible levels of expression was observed. It may be noted that the parental EF1prtTA cell line chosen from the initial screen (previous section, Fig 3) had not exhibited a very high level of fold inducibility in transient assays. However, on introduction of a Tc-operator regulatable construct, in a stably integrated form, high levels of Tc-dependent

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induction was observed in individual clones (Fig. 4A compare lanes 1, 3, 9, 10 and 17, induced and uninduced level and similarly Fig. 4B lanes 1, 4 and 9). Overall, the frequency, variability and basal to induced levels obtained in various clones closely parallels that reported for the Tc-system (Gossen et al., 1994; Gossen and Bujard, 1992; Gossen et al., 1995).

DISCUSSION

Inability to support continual strong expression from a given type of promoter, specifically those of viral origin has been documented for certain cell types (Gorman et al., 1985; Hasegawa et al., 1990; Li et al., 1992; Miller and Rizzino, 1995; Sleight, 1987). The primary goal of this work is to reduce a significant and hitherto unaddressed variable in successfully establishing Tc-inducible cells. Expression of the Tc-operator expression construct, pUHD 10-3 (Gossen and Bujard, 1992) or its derivatives, into which the cDNA of interest is usually cloned, is ultimately dependent on expression of the tTA or rtTA gene product. Preventing or avoiding TA cDNA expression is shut down, during or subsequent to establishing a cell line, a variable that is likely to be cell type associated (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995) should considerably enhance success rates. To achieve steady and adequate levels of the TA cDNA expression, relatively independent of temporal factors, cell-type, cell physiology status and cell passage number, we replaced the CMV IE promoter enhancer with the cellular EF-1 α promoter (Goldman et al., 1996; Kim et al., 1990; Wakabayashi-Ito and Nagata, 1994). Experience in using pUHD 17-lneo (Gossen et al., 1995) indicated that while activity and inducibility in transient assays using sensitive detection methods with luciferase reporters worked reasonably well, we failed to generate cells showing any level of activity of the gene of interest after clonal selection of

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individual lines, despite presence of expression construct DNA, in the genome using Southern analysis (data not shown).

Numerous modifications of the basic Tc-regulatable system have been reported in the literature directed toward enhancing performance. Several alternative promoters have been utilized to drive expression of the TA cDNA. Many of these are based on the requirement for tissue or species specific expression in plants (Weinmann et al., 1994), , Drosophila (Bieschke et al., 1998) or mice, (Bohl et al., 1997; Dhawan et al., 1995; Faiss et al., 1997; Hennighausen et al., , 1995; Hoffmann et al., 1997; Holwell et al., 1997; Li et al., 1992; Liang et al., 1996; Miller and Rizzino, 1995; Thompson and Myatt, 1997). Another modification of the TA expressing construct involves use of bi- or multi-cistronic plasmid constructs which drives expression, through oppositely oriented promoters, of both TA-cDNA and Tc-operator regulated cDNAs, mainly to circumvent two rounds of transfection of separate plasmids (Baron et al., 1995 Fussenegger et al., 1997; Liang et al., 1996; Schultze et al., 1996; Weinmann et al., 1994). However they are based on one or a combination of viral promoters with accompanying drawbacks mentioned above. Multi-cistronic single retroviral or combinations of two or more retroviruses expressing different components has also been constructed (Bohl et al., 1997; Hofmann et al., 1996; Kringstein et al., 1998; Paulus et al., 1996; Rossi et al., 1998). These overcome the barrier of gene delivery into cells but again expression is often based on viral promoter sequences, prone to possible shutdown in some cell types. The relatively complex steps involved in making a virus for a given cDNA of interest including the intricate cloning strategies due to large vector size and investment in time, somewhat offsets the advantages they present over classical DNA transfection approaches. Making retroviral vectors is presently restricted to a relatively small proportion of laboratories and safety

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concerns impose limitations of use in several setups. Therefore, while these vectors hold considerable promise, the likelihood of a major shift over to their usage from widespread DNA transfection approaches may only be in the long term. The relevance of improved plasmid vectors is therefore still strong.

A generally applicable modification to the original TA-expression construct involved expression of both TA-cDNA and exogenous cDNA under regulation of Tc-operator sequences (Liang et al., 1996; Shockett et al., 1995). The rationale being that, exquisite regulation with very high inducibility could be built into a system when both the activator molecule and the regulatable gene of interest are under control of the same inducer through an autoregulatory loop. Unfortunately, it appears that the high levels of tTA protein produced as a result of induction results in toxic side effects in cells (Gallia and Khalili, 1998; Gossen and Bujard, 1992) most likely due to interference in cellular metabolism by the acid activation domain of the HSV, VP16 protein present in TA-proteins. This could be an additional reason why certain cell types apparently shut down expression of TA-cDNA after extended periods time. Alternatively, cells strongly expressing TA proteins might be at a selective disadvantage, particularly in cells with a long doubling time due to accumulation of toxic levels of TA protein. While we can only speculate about the true reason for the apparent loss of TA expression, it appears that switching over to the EF-1 α expression cassette is able to balance out and overcome these problems.

The conclusion is based on observations over periods of time, extending to almost twelve months in the case of certain EFlprtTA cells lines such as those established in HO-1 melanoma. The parental HO-1 EFlprtTA cell line was made and initially analyzed over a period of time (> 60 days) before

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being expanded and frozen for future use. These parental cells were used to establish inducible Jun B and Mda-7 expression (Fig 4, A and B) that showed functional levels of TA-expression and inducible properties after being thawed out
5 several months and passage numbers subsequent to when the line had initially been established and frozen. This line and others (Fig. 3) continue to retain Tc-responsive properties and were all maintained in the absence of antibiotic selection, indicating that expression of the rtTA cDNA
10 continued irrespective of lack of positive selective pressure, passage number and time elapsed between introduction and integration of the plasmid DNA and final usage. Overall, following modification of the expression construct for the rtTA cDNA we have demonstrated that it had
15 enabled us to significantly enhance the likelihood of establishing cell lines that are Tc-regulatable. It appeared that positive clones were obtained at higher frequencies than previously reported and that consistent expression and clonal stability over an extended period of time was accomplished.
20 Based on these observations we conclude that the modified EF1prtTA presents a useful reagent with broad applicability in establishing Tc-regulatable cells.

**Generation of Transgenic mice expressing the rtTA cDNA under
25 control of the EF-1 α promoter**

Experiments to obtain expression of the rtTA protein in all tissues of mice, utilizing transgenic technology, are presently in progress. The EF-1 α gene and its promoter are ubiquitously expressed in all animal tissues and is therefore
30 a suitable expression system to achieve this goal. A transgenic expression cassette, consisting of the human EF-1 α gene promoter linked to the rtTA cDNA has already been constructed and functionally tested in rat, mouse and human cell lines (described in the literature as pEF1prtTA
35 (Gopalkrishnan et al., Nuc. Acids Res. 27:4775-4782, 1999 and

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references therein)). Standard procedures in the generation of transgenic mouse lines was performed. In brief, microinjection of pEF1prtTA into pronuclei of fertilized mouse eggs was carried out and these were implanted into

5 psuedo-pregnant female mice. These manipulations resulted in a final litter of three mice which were analyzed for presence of the transgene in genomic DNA, derived from tail-tip samples by genomic Southern blot analysis using a radioactively labeled rtTA cDNA probe. This analysis

10 revealed that one of the three founder mice was positive for the transgene since it displayed an appropriate sized band as detected by autoradiography. This founder, a female, has been subsequently crossed with wild type male mice to generate F1 progeny. Analysis of tail-tip DNA from the F1

15 generation has permitted us to determine whether the founder possess the capacity to transmit the transgene. Southern blot analysis of tail-tip DNA from 15 F1 generation indicated that eight (8) mice were positive for the transgene, confirming that the original founder animal had the capacity

20 to transmit the inserted gene. Subsequent to our successful generation of transgenic mouse lines, we are presently in a position to breed additional animals and begin extensive expression analysis of the transgene to determine level of expression. This will be carried out on F1 or later

25 generation mice, while maintaining the original founder until we are certain that stable expressing lines can be generated from progeny for future use and distribution. These mouse lines can be used to generate mice that can inducibly express specific genes under regulation of tetracycline to study the

30 in vivo effect of specific genes in animals or screen for anti-tumoral or other pharmacological effects of drugs or small molecules.

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Construction of pEF1ptTA, an expression vector expressing tTA, tetracycline repressor under regulation of the EF-1 α promoter for significantly increasing success in establishing stable cell lines with consistent expression.

5 An EcoRI-BamHI fragment containing the tTA open reading frame was isolated from pUHD 15-1. This fragment was cloned directionally into the mammalian expression vector pCDEF3 at the 5' EcoRI and 3' XbaI sites of the vector multiple cloning site to generate the final construct, termed EF1ptTA.

10 Ligation of the 3' XbaI site of pCDEF3 and the Bam HI site of the fragment was possible after Klenow filling the overhangs to make them blunt-ended. This modified vector places the tTA gene under direct transcriptional control of the human EF-1 α promoter. The construct was confirmed by

15 restriction enzyme and DNA sequencing analysis. Functional testing in rodent and human cell lines is presently underway and will be performed essentially as described for pEF1prtTA. Compared to the earlier construct (pEF1prtTA) wherein gene expression is induced in the presence of the inducer

20 (tetracycline or doxycycline), the present construct is active in the absence of tetracycline or doxycycline and gene expression is shutdown in the presence of these reagents. Both plasmids may be used in setting up inducible gene expression systems in cell lines or mice and the choice will

25 be dependent on whether one desires to grow cells in the presence or absence of the chemical agent.

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1. A vector comprising:
 - a) a human Protein Translation Peptide Elongation Factor-1 α promoter;
 - b) a nucleic acid encoding a tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of the promoter;
 - c) a tetracycline inducible operator binding element under the control of the nucleic acid encoding the transactivator, and
 - d) a gene of interest under the control of the promoter.
2. The vector of claim 1, wherein the vector is a plasmid.
3. The vector of claim 1, wherein the vector is as set forth in figure 1.
4. An isolated cell comprising the vector of claim 1.
5. The cell of claim 4, wherein the cell is from a cell line.
6. The cell of claim 5, wherein the cell line is HeLa (human cervix), HO-1 (human melanoma), MCF-7 (human breast), PC3 (human prostate) or DU-145 (human prostate).
7. The cell of claim 4, which consistently expresses tetracycline repressor.
8. A cell comprised of Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein

Translation Peptide Elongation Factor-1 α promoter.

9. A non-human animal comprising the vector of claim 1.
10. The animal of claim 9, wherein the animal is a mouse.
11. A method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising:
 - a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion.
 - b) generation of Protein Translation Peptide Elongation Factor-1 α promoter vector, by restriction enzyme digestion;
 - c) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs;
 - d) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by Klenow fragment mediated blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' XbaI end of Protein Translation Peptide Elongation Factor-1 α promoter vector; and
 - e) blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor-1 α promoter vector expressing reverse tetracycline controlled transactivator.
12. The method of claim 11, wherein the fragment of 11(a) is an Eco RI-BAM HI fragment.

13. The method of claim 11, wherein the mammalian expression vector of 11(b) is pCDEF3.
14. The method of claim 11, wherein the cloning of 11(a) is at the 5' *Eco* RI and 3' *BAM* HI sites.
15. The method of claim 11, wherein the ligation of 11(c) is at the 5' *Eco* RI site of pCDEF3.
16. The method of claim 11, wherein the ligation of 11(d) is at the 3' *Xba*I site of pCDEF3.
17. A vector generated by the method of claim 11.
18. A method for screening for an anti-tumor drug which comprises administering to a transgenic non-human animal a drug wherein the animal inducibly expresses or represses expression of a gene of interest under regulation of tetracycline or deoxycycline and wherein the gene of interest is associated with cancer, and determining whether the animal develops a tumor thereby screening for an anti-tumor drug.
19. A method for expressing a gene of interest which comprises contacting the cell of claim 4 with an inducer of the tetracycline inducible operator binding element so as to cause the cell to express the gene of interest.
20. The method of claim 19, wherein the inducer is tetracycline or deoxycycline.

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/00, 5/00, 15/63, C12P 21/06, C12Q 1/68, A01K 67/00	A1	(11) International Publication Number: WO 00/55310 (43) International Publication Date: 21 September 2000 (21.09.00)
(21) International Application Number: PCT/US00/06862 (22) International Filing Date: 15 March 2000 (15.03.00) (30) Priority Data: 09/268,303 15 March 1999 (15.03.99) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/268,303 (CIP) Filed on 15 March 1999 (15.03.99) (71) Applicant (for all designated States except US): THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): FISHER, Paul, B. [US/US]; 15 Gordon Place, Scarsdale, NY 10583 (US). GOPALKRISHNAN, Rahul [IN/US]; 302 West 79th Street, Apartment 2A, New York, NY 10024 (US). (74) Agent: WHITE, John, P.; Cooper & Dunahm LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: IMPROVED EXPRESSION VECTOR FOR CONSISTENT CELLULAR EXPRESSION OF THE TET ON REPRESSOR IN MULTIPLE CELL TYPES		
(57) Abstract <p>The present invention provides a vector comprising Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor-1 α promoter. In addition, the invention provides a method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising: (a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion, (b) generation of Protein Translation Peptide Elongation Factor-1 α promoter vector, by restriction enzyme digestion, (c) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs, (d) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by Klenow fragment mediated blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' XbaI end of Protein Translation Peptide Elongation Factor-1 α promoter vector and (e) blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor-1 α promoter vector expressing reverse tetracycline controlled transactivator.</p>		

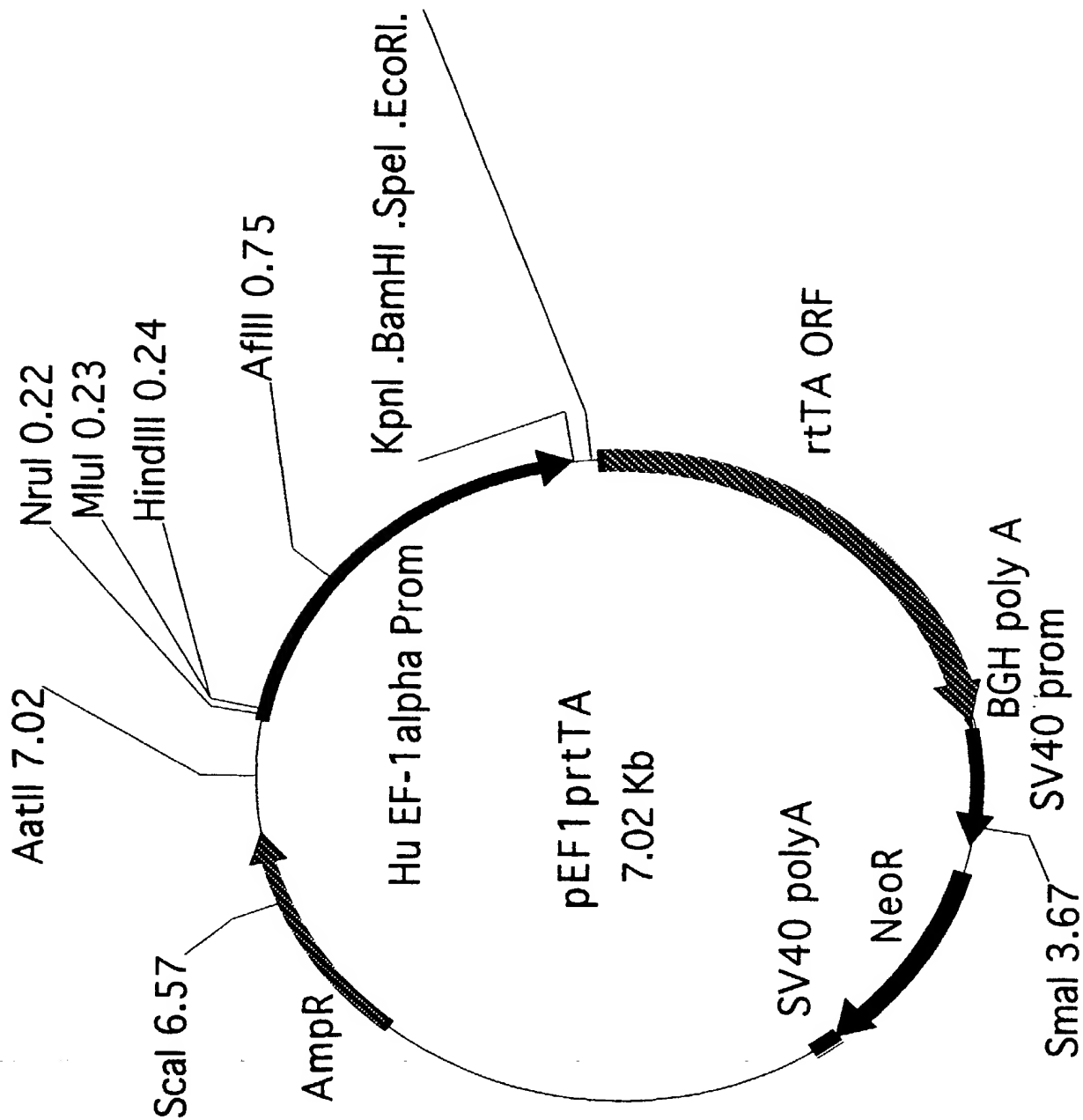
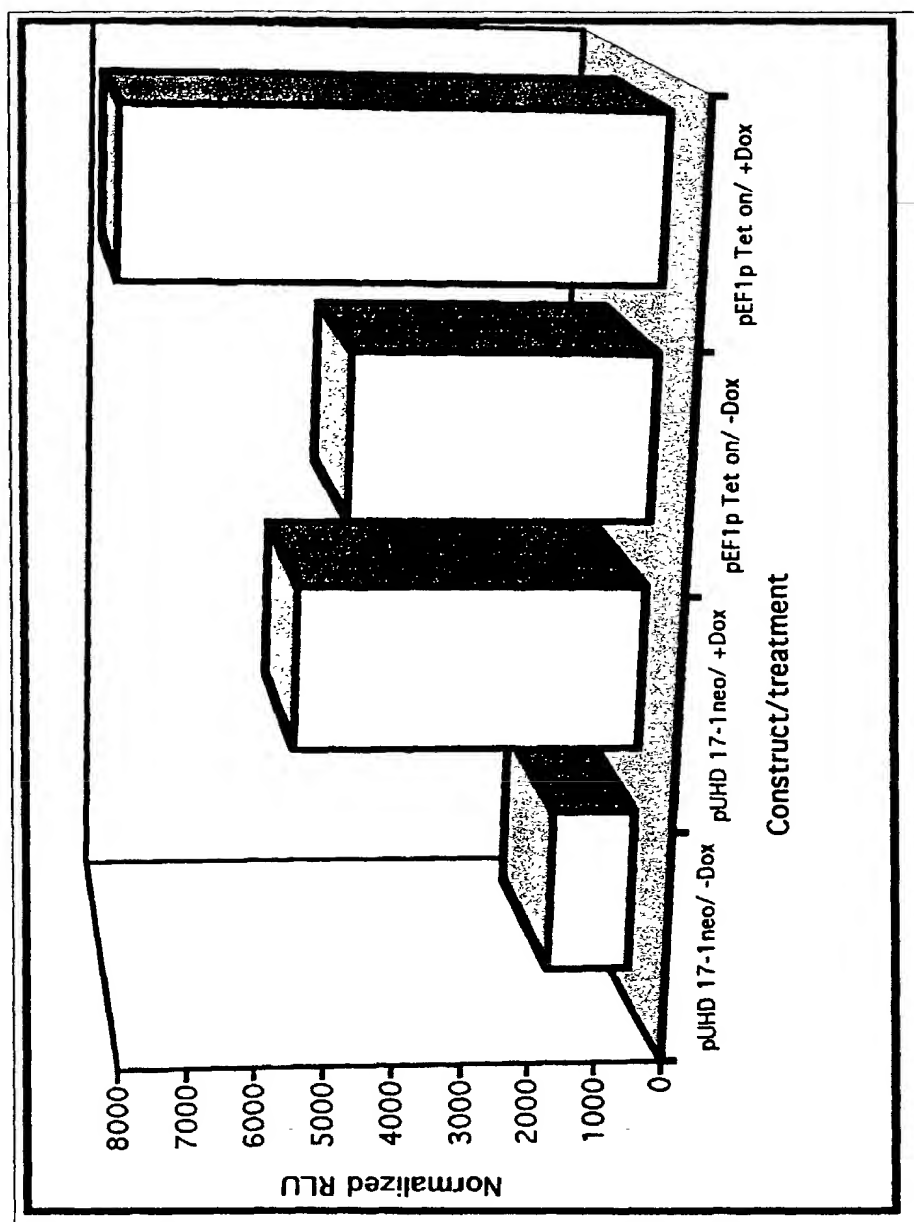


FIG. 1

FIG. 2



09/937165

FIG. 3A

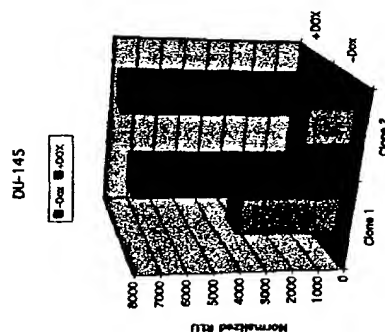


FIG. 3B

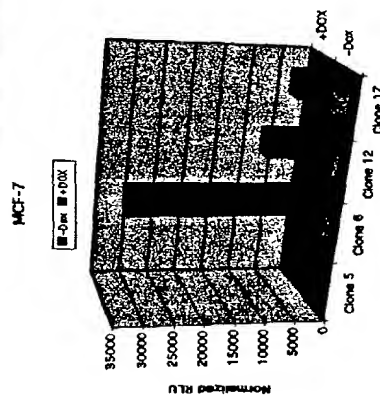
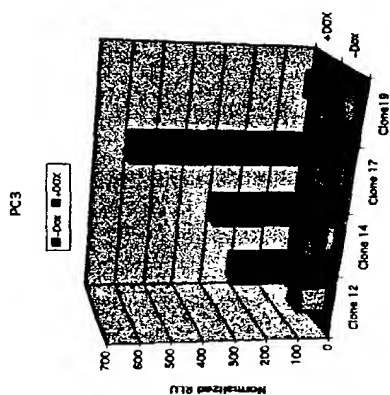


FIG. 3C



HeLa

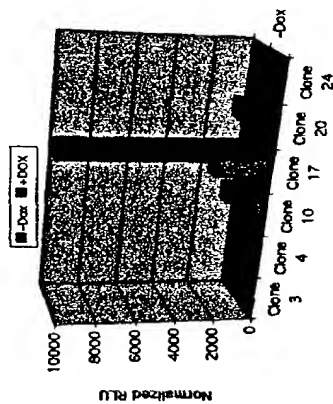


FIG. 3D

HO-1

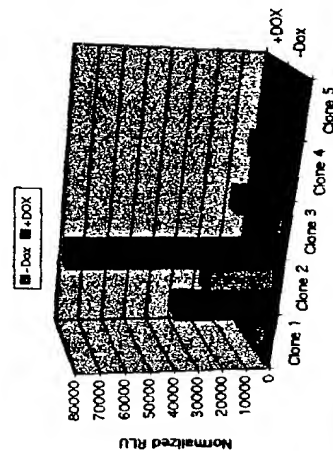


FIG. 3E

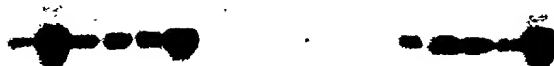
Figure 3

FIG. 4A

A

Mda-7

1 1' 2 2' 3 3' 4 4' 5 5' 6 6' 7 7' 8 8' 9 9'



10 10' 11 11' 12 12' 13 13' 14 14' 15 15' 16 16' 17 17'



FIG. 4B

B

Jun B

1 1' 2 2' 3 3' 4 4' 5 5'

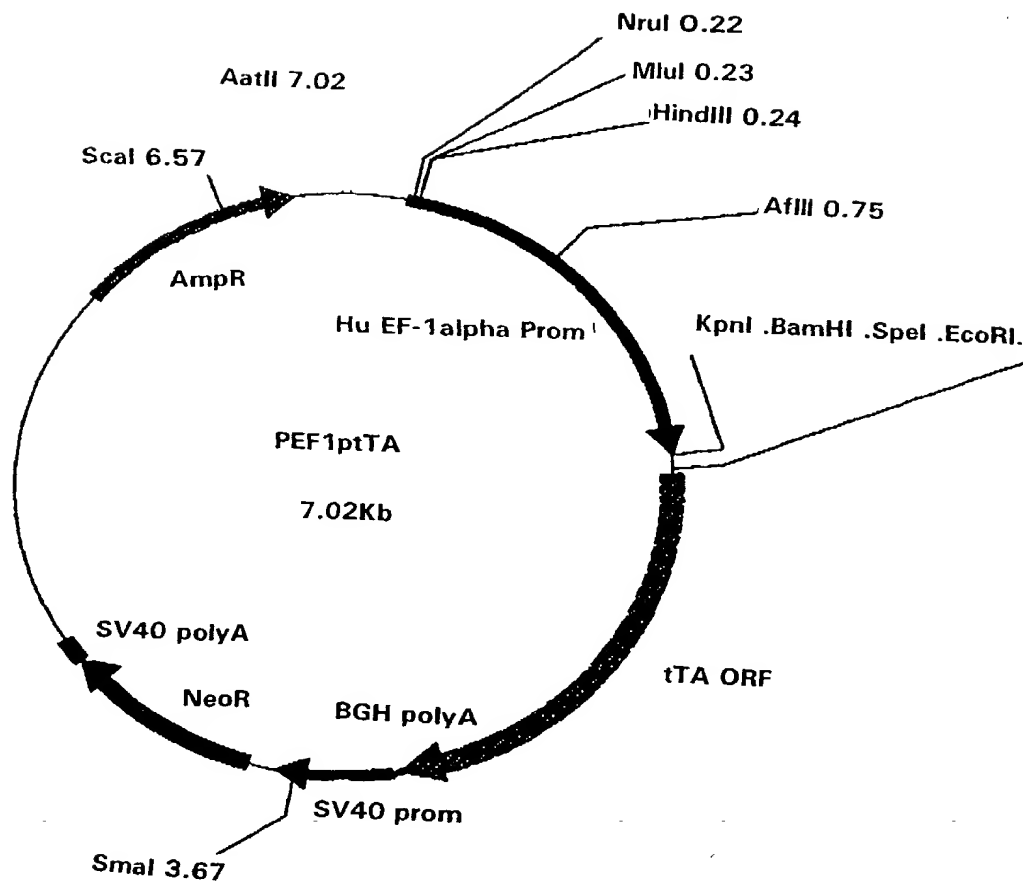


6 6' 7 7' 8 8' 9 9'



Figure 4

Figure 5



**COMBINED DECLARATION
AND POWER OF ATTORNEY**

(Original, Design, National Stage of PCT, Divisional, Continuation or C-I-P Application)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

IMPROVED EXPRESSION VECTOR FOR CONSISTENT CELLULAR EXPRESSION OF THE TET ON REPRESSOR IN MULTIPLE CELL TYPES

This declaration is of the following type:

- ☐ original
- ☐ design
- ☒ national stage of PCT.
- ☐ divisional
- ☐ continuation
- ☐ continuation-in-part (C-I-P)

the specification of which: *(complete (a), (b), or (c))*

- (a) ☐ is attached hereto.
- (b) ☐ was filed on as Application Serial No. .
- (c) ☒ was described and claimed in PCT International Application No. PCT/US00/06862 filed on 15 March 2000 and was amended on *(if applicable)*.

Acknowledgement of Review of Papers and Duty of Candor

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56.

☐ In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.98.

Priority Claim

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT International Application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT International Application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application on which priority is claimed

(complete (d) or (e))

- (d) ☐ no such applications have been filed.
- (e) ☒ such applications have been filed as follows:

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			
COUNTRY	APPLICATION NO	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
			PRIORITY CLAIMED UNDER 35 USC 119 <input type="checkbox"/> YES <input type="checkbox"/> NO <input type="checkbox"/>
			<input type="checkbox"/> YES <input type="checkbox"/> NO <input type="checkbox"/>
			<input type="checkbox"/> YES <input type="checkbox"/> NO <input type="checkbox"/>

ALL FOREIGN APPLICATION[S], IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			
COUNTRY	APPLICATION NO	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
			PRIORITY CLAIMED UNDER 35 USC 119 <input type="checkbox"/> YES <input type="checkbox"/> NO <input type="checkbox"/>
			<input type="checkbox"/> YES <input type="checkbox"/> NO <input type="checkbox"/>
			<input type="checkbox"/> YES <input type="checkbox"/> NO <input type="checkbox"/>

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date

Claim for Benefit of Earlier U.S./PCT Application(s) under 35 U.S.C. 120

(complete this part only if this is a divisional, continuation or C-I-P application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

<u>PCT/US00/06862</u> (Application Serial No)	<u>15 March 2000</u> (Filing Date)	<u>Pending</u> (Status) (patented, pending, abandoned)
<u>09/268,303</u> (Application Serial No)	<u>March 15, 1999</u> (Filing Date)	<u>Pending</u> (Status) (patented, pending, abandoned)

Power of Attorney

As a named inventor, I hereby appoint Robert Neuner, Reg. No. 24,316; Richard G. Berkley, Reg. No. 25,465; Bradley B. Geist, Reg. No. 27,551; James J. Maune, Reg. No. 26,946; John D. Murnane, Reg. No. 29,836; Henry Tang, Reg. No. 29,705; Robert C. Scheinfeld, Reg. No. 31,300; John A. Fogarty, Jr., Reg. No. 22,348; Louis S. Sorell, Reg. No. 32,439; Rochelle K. Seide Reg. No. 32,300; Gary M. Butter, Reg. No. 33,841; Lisa B. Kole, Reg. No. 35,225; Anthony Giaccio, Reg. No. 39,684; Carmella L. Stephens, Reg. No. 41,328; and Alicia Russo, Reg. No. 46,192 of the firm of BAKER & BOTTS, L.L.P., with offices at 30 Rockefeller Plaza, New York, New York 10112, as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

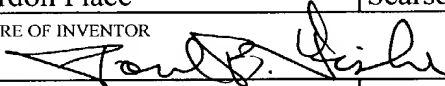
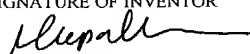
SEND CORRESPONDENCE TO: BAKER & BOTTS, L.L.P. 30 ROCKEFELLER PLAZA, NEW YORK, N.Y. 10112 CUSTOMER NUMBER: 21003	DIRECT TELEPHONE CALLS TO: BAKER & BOTTS, L.L.P. (212) 705-5000
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the

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knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00 FULL NAME OF SOLE OR FIRST INVENTOR	LAST NAME <u>Fisher</u>	FIRST NAME <u>Paul</u>	MIDDLE NAME <u>B.</u>	
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DATE <u>7/24/02</u>	SIGNATURE OF INVENTOR 			
2-00 FULL NAME OF SECOND JOINT INVENTOR, IF ANY	LAST NAME <u>Gopalkrishnan</u>	FIRST NAME <u>Rahul</u>	MIDDLE NAME <u>V.</u>	
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DATE <u>7/24/02</u>	SIGNATURE OF INVENTOR 			

Check proper box(es) for any added page(s) forming a part of this declaration

- ☐ Signature for ninth and subsequent joint inventors. Number of pages added _____.
- ☐ Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor.
Number of pages added _____.
- ☐ Signature for inventor who refuses to sign, or cannot be reached, by person authorized under 37 CFR 1.47.
Number of pages added _____.